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EFFECT OF TEMPERATURE, pH AND NaCl ON NISIN ACTIVITY AGAINST *LACTOBACILLUS FRUCTIVORANS*

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Abstract:
The aim of this work was to determine the influence of pH, sodium chloride concentration and the combination of different temperatures and periods of exposure on the antimicrobial activity against *Lactobacillus fructivorans*.
Turbidity of the different system conditions was monitored in order to establish the microorganism growth. The effect of temperatures ranging from 60 to 120ºC during periods of 10 to 60 minutes

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was evaluated. Nisin activity was only changed by temperatures ranging from 100 to 120ºC, in several exposure times. A pH range between 3.5 and 7 was tested. The highest inhibitory effect was recorded for neutral pH and lightly acidic conditions.
Sodium chloride concentrations studied were 1 and 2 % w/v. The latter value helped to enhance the inhibitory effect of the bacteriocin. These results show the influence that the studied factors exert on nisin activity against *L. fructivorans* and suggest that they have to be taken into account when nisin is to be used as a biopreservative in food.

Resumen
El presente trabajo tuvo como objetivo determinar la influencia del pH, concentración de cloruro de sodio y la combinación de diferentes temperaturas y períodos de exposición sobre la actividad antimicrobiana de nisina sobre una bacteria alterante *Lactobacillus fructivorans.*
El crecimiento del microorganismo bajo diferentes condiciones fue determinado por la presencia de turbidez. Se evaluó el efecto de la temperatura en un rango de 60 a 120ºC durante períodos de 10 a 60 minutos. La actividad de la nisina fue afectada solamente por temperaturas de 100 y 120ºC, a cualquier tiempo de exposición considerado. Los ensayos se realizaron con un rango de pH entre 3,5 y 7. El mayor efecto inhibitorio fue a pH neutro y levemente ácido. Las concentraciones de cloruro de sodio fueron de 1 y 2% p/v. Sólo a la mayor concentración se ve un incremento en la actividad inhibitoria de la bacteriocina. Estos resultados muestran la influencia que los factores estudiados ejercen sobre la actividad de la bacteriocina frente a *L. fructivorans* e indican que tales factores deben ser tenidos en cuenta cuando la nisina sea usada en la preservación de alimentos

Introduction

Two essential factors involved in the preservation of food are acidification and thermal processing. These steps are crucial to control microbial growth and hence, to assure the safety and stability of a food along its shelf-life. However, changes in pH and severe thermal treatments can alter food organoleptic characteristics and modify the efficiency or functionality of some additives.

Nowadays, consumers are particularly aware of the health concerns regarding food additives; the health benefits of “natural” and “traditional” foods, processed with no added chemical preservatives, are becoming more and more attractive. Thus, in the last decades, due to consumer demand for higher quality and naturalness of foods, as well as strict government requirements for guarantees of food safety, food producers have faced conflicting challenges [1]. In this context, bacteriocins may be considered as natural preservatives which meet consumers’ expectations.

Bacteriocins have often been mooted as potentially valuable biological tools to improve the food safety and reduce the prevalence of foodborne illnesses. It is usually suggested that bacteriocins should not be used as the primary processing step or as a barrier to prevent the growth or survival of pathogens, but rather that they could provide an additional hurdle to reduce the likelihood of foodborne disease. Thermal processing is used extensively within the food manufacturing process and can have adverse effects on the bio-active capability of a bacteriocin, potentially rendering it less effective. The chemical and physical properties of a food, e.g. pH, and fat content, can also have a significant role in the suitability of a particular bacteriocin [2].

Nisin is widely used as a preservative in the food industry and has a good potential for use in health care products such as toothpaste and skin care products [3]. Nisin is a 34 residue long peptide bacteriocin produced by strains of *Lactococcus lactis* subsp. lactis, and it exerts bactericidal effects against gram-positive bacteria. The compound is commercially available and has successfully been used as a biopreservative in dairy and meat products [4]. In the United States, nisin has received GRAS (generally recognized as safe) status and it is approved for use in some processed cheese spreads to prevent the outgrowth of clostridial spores and toxin production. In addition, nisin has been used to inactivate thermophilic spoilage organisms in canned goods and to extend the shelf life

of milk and dairy products Nisin shows antimicrobial activity against a wide range of Gram-positive bacteria but shows little or no activity against Gram-negative bacteria, yeasts or moulds [5]. Gram-positive spore formers i.e. *Bacillus, Clostridium* spp. are particularly sensitive to nisin, with spores being more sensitive to nisin than vegetative cells. Such an antimicrobial spectrum has resulted in nisin being used as a commercial preservative in products which by their nature cannot be fully sterilized but only pasteurized during their production. Nisin also shows activity against lactic acid bacteria. As such bacteria are often capable of growing at low pH, nisin can be used as a preservative in low pH foods that are not heat processed, such as salad dressings and alcoholic beverages [6].

Henning and coworkers [7] presented evidence showing that the antimicrobial effect of nisin is caused by interaction of nisin with the phospholipid component of the cytoplasmic membrane. They demonstrated that isolated cytoplasmic membrane fragments could antagonize the inhibitory effect of nisin, and that nisin would combine with phospholipids to form nisin-phospholipid complexes. The initial electrostatic attraction between the target cell membrane and the bacteriocin peptide is thought to be the driving force for subsequent events. While many bacteriocins have been shown to induce pore formation in sensitive microorganisms, the mechanism of action of nisin has been studied in the greatest detail. Nisin forms pores that disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death [2].

The chemical composition and the physical conditions of food can have a significant influence on the activity of the bacteriocin [8]. In food matrices the bacteriocin activity may be modified by: (i) changes in solubility and charge of the bacteriocins, (ii) binding of the bacteriocins to food components, (iii) inactivation by proteases, and (iv) changes in the cell envelope of the target organisms as a response to environmental factors [9]. Rogers and Montville [10] reported that temperature, phospholipidic content and pH affect nisin inhibitory activity against *Clostridium botulinum* in model food systems.

Lactobacillus fructivorans, a heterofermentative lactobacilli, Gram-positive bacteria, was first isolated from spoiled salad dressing and has been reported to spoil acidic food or ethanol-containing sources, mainly mayonnaise, salad dressings, vinegar preserves, sake, dessert wines, and aperitifs [11]. Thus, *L. fructivorans* shows a high tolerance to acidic environments, where acetic or citric acids can be present, as well as a remarkably resistance to sorbic (or benzoic) acid and/or ethanol. Castro and coworkers [12] studied the effect of potassium sorbate, nisin, Tween® 20 and oil level on the survival of *L. fructivorans* in model salad dressings finding that the bacteria is resistant to the antimicrobial activity of sorbate, but it showed to be sensitive towards nisin at pH 3.5.

Due to bacteriocin activity differs from one sensitive species to another, bacteriocin affinity towards specific species and strains must be studied. As far as our knowledge, for the time being, the application of nisin to prevent *Lactobacillus fructivorans* spoilage has not been extensively studied. As a consequence, this study was devised to evaluate the influence of factors like temperature and time of exposure, pH and sodium chloride concentration on the activity of nisin against *L. fructivorans.*

Materials and methods

Strain and culture conditions

L. fructivorans CRL941 from CERELA (Centro de Referencia para Lactobacilos; Tucumán, Argentina) was kept frozen at -18°C in MRS broth + glycerol (20%v/v). It was activated in MRS broth at 32ºC during 48 h and subcultured twice at 24 h at the same temperature.

Bacteriocin

Nisin (Nisaplin, $1x10^6$UI/g) was a donation from Aplin & Barrett Ltd. Nisin was solubilized with sterile solution of HCl 0,02N and 0,75% w/v NaCl, in a laminar flow (HEPA filter of 0,22µm), to obtain a solution of 500 ppm.

Determination of bacteriocin activity

Inhibitory activity of the bacteriocin was considered positive when absence of turbidity was visually observed in the tubes with broth where *L. fructivorans* was inoculated. As blank controls, tubes were prepared for each treatment with nisin but without the microorganism. All the experiments, i.e. every treatment assayed, were performed in triplicate and the results are displayed as the mean values of the experiments**.**

In order to express the visual variations perceived in turbidity, an arbitrary scale was assigned to the different degrees of turbidity observed along the experiences.

Effect of temperature

Sterilization by autoclaving at 120°C for 20 min. In order to test the effect of sterilization on nisin activity, tubes with 5 ml MRS broth (Biokar Diagnostics, Beauvais, France) were used. Nisin solution was added to these tubes to obtain concentrations of 20.0; 35.0; 50.0; 65.0; 80.0 and 100.0 ppm. After autoclaving, the systems were inoculated with 50 µl of *L. fructivorans* and were incubated at 32°C during 24 h. Control tubes were prepared in the same fashion, avoiding the autoclaving step.

Heating: the effect of heating on nisin activity was evaluated at different time intervals for each of the temperatures chosen: 100ºC; 80ºC and 60ºC. Tubes with 5 ml MRS broth were added with nisin solution in order to get concentrations of: 1.0; 2.5; 4.0; 6.0 y 10.0 ppm. These tubes were exposed to the different temperature/time intervals and then they were inoculated with 50 µl of an active culture of *L. fructivorans*. Afterwards, they were incubated at 32ºC for 24 h.

Samples were exposed to: a) 100ºC at 10, 20 and 30 minutes; b) 80ºC at 15, 30 and 45 minutes; c) 60ºC at 20, 40 and 60 minutes. The procedure was done in a heating bath (Büchi, Switzerland).

The control sample consisted of a tube with 5 ml MRS broth added with the corresponding nisin solution without heating.

Effect of pH

The pH range tested was 3.5, 4.0, 4.5, 5.0; 6.0 and 7.0. The values 3.5 and 4.5 were chosen based on the range of *Lactobacillus fructivorans'* optimal growth. To evaluate the pH influence on nisin activity, 5 ml of MRS broth were adjusted with appropriates volumes of 50% w/v citric acid to obtain pH values of 3.5, 4.0, 4.5, 5.0; 6.0 and 7.0 with a pHmeter (Oaklon pH 510 series) after autoclaving at 120°C for 20 min.

Nisin stock solution of 500 ppm was added to MRS broth in order to get concentrations of 2.5; 4.5; 7.0; 12.0 y 20.0 ppm. The tubes were inoculated with 50 µl of an active culture of *L. fructivorans* and then they were incubated at 32ºC for 24 h.

Effect of NaCl concentration

Tubes containing sterile MRS broth were added with the convenient volume of nisin stock solution in order to get the following concentrations: 4.0; 6.0; 8.0 and 10.0 ppm. Sterile concentrated solution of sodium chloride (35 % w/v) was poured into the tubes to get

concentrations of 1.0 and 2.0 % w/v. The tubes were inoculated with 50 µl of an active culture of *L. fructivorans* and then they were incubated at 32ºC for 24 h.

Salt concentrations were chosen so as to mimic salt content of several foods such as salad dressings and meat products.

All the experiments were performed in triplicate and the results are displayed as the mean values of the experiments**.**

Results

Effect of temperature on nisin activity

Sterilization.

Samples subjected to this thermal treatment showed differences compared to the respective control samples. At all the different concentrations tested, nisin was effective for the inhibition of *L. fructivorans* prior to sterilization. On the contrary, nisin lost its effectiveness after thermal processing, being necessary concentrations as high as 50 ppm to inhibit bacterial growth.

Heating.

As Table 1 shows, nisin activity was modified by the treatment at 100ºC at almost all the time intervals tested. Growth was observed at 10 min and 10 ppm showing that this condition is not enough to alter the nisin activity. However at 60 and 80ºC its effectiveness was not changed compared to the control systems. The growth was followed by visually examining the turbidity of the broth.

Table 1. Effect of temperature/time of exposure on the activity of nisin against *L. fructivorans*

Temperature [ºC]	C	60			80			100		
t [minutes]		20	40	60	15	30	45	10	20	30
Nisin concentration [ppm]										
1.0	+	+	+	+	+	+	+	+	+	+
2.5	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+
6.0	-	-	-	-	-	-	-	+	+	+
10.0	-	-	-	-	-	-	-	-	+	+

(+) Growth$_{(1)}$ (-) No growth C = control

(1) The growth was followed by visually examining the turbidity of the broth.

Effect of pH on nisin activity

Nisin activity is dependent upon pH of the medium, as it can be seen in table 2. This activity also depends on the concentration of the bacteriocin. The higher the concentration and pH value, the better the performance of the natural antimicrobial substance on the inhibition of *L. fructivorans.* For the lowest level of nisin assayed, 2.5 ppm, bacterial growth was observed at all the pH values tested. Concentrations of 4.5, 7.0 and 12.0 ppm were effective at pH values of 6.0 and 7.0, showing no differences between them. Twenty ppm of nisin inhibited bacterial growth at pH values of 5.0, 6.0 and 7.0, showing less turbidity in the tubes corresponding to the rest of the pH values. This

reduction in the turbidity observed in the tubes corresponding to 20.0 ppm of nisin can be noticed comparing them with the other concentrations of nisin tested. Table 2 shows the variations on turbidity values observed when nisin at different concentration were added to MRS broth at different pH values.

Table 2 Effect of pH on nisin effectiveness against *L. fructivorans*

pH	Nisin concentration [ppm]				
	2.5	**4.5**	**7.0**	**12.0**	**20.0**
3.5	++	++	++	++	+
4.0	++++	++++	++++	++++	+++
4.5	++++	++++	++++	++++	++
5.0	+++	+++	+++	+++	-
6.0	++	-	-	-	-
7.0	+	-	-	-	-

Turbidity (+) degree (-) No turbidity

Effect of NaCl concentration on nisin activity

The addition of NaCl, even at the higher concentration tested (2% w/v,) did not change the inhibitory activity of nisin against *L. fructivorans*. The necessary concentration of the bacteriocin to inhibit bacterial growth was 6 ppm, the same as for the control samples. The lowest concentration tested (4 ppm) helped to retard bacterial growth, at both levels of NaCl, giving less turbidity which denotes a diminished bacterial load.

Discussion

The major functional limitations for the application of bacteriocins in foods are their relatively narrow activity spectra and moderate antibacterial effects. Moreover, they are generally not active against gram-negative bacteria. To overcome these limitations, more and more researchers use the concept of hurdle technology to improve shelf-life and enhance food safety. It is well documented that nisin enhances thermal inactivation of bacteria, thus reducing the treatment time and resulting in better food qualities [13]. The application of hurdle technology implies a well understanding of every hurdle employed, including the effects that each particular hurdle exerts among the others. In this sense, the addition of nisin as a biopreservative into a food system requires this knowledge.

Considering the proteinaceous nature of nisin, it is expected to be modified by heat treatments during the manufacturing process of food. The value of ph at which sterilization and heating treatments were done was 6.4±0.2, being the one corresponding to the pH of the culture media employed, i.e. MRS broth. The necessary amount of nisin required to inhibit bacterial growth were higher for those systems with nisin subjected to sterilization, compared to those for which the culture broth was sterilized before nisin was added. These results are in agreement with Delves-Broughton *et al.* [6] who stated that nisin becomes less stable to heating and losses significantly its activity when it is heated at high temperatures in the pH range 5-7.

Heating at 60, 80 and 100ºC, instead of 120ºC at 1 atm, was less harmful since the differences with the control samples were only appreciable at high concentrations of the bacteriocin during all the times of exposure assayed. Delves-Broughton and coworkers [6] proved that pasteurization processes retain less than the 80 % of nisin activity, in this study nisin was more stable showing to retain its activity during exposure at temperatures of 60 and 80ºC, compared to the control samples.

Nisin inhibitory effect was favored by raising pH levels, reaching its highest antimicrobial activity at pH values of 6.0 and 7.0. However, it is important to remark that turbidity was declined at the lower pH value studied (3.5) for all the concentrations of nisin tested. *L. fructivorans* showed to grow well at ph 4-4.5, which is in agreement with the pH values of the food that this bacterium usually spoils.

Researchers showed that nisin enhanced its activity towards *Listeria monocytogenes* at low pH values [14]. The antibacterial activity increase at low pH is usual [15], and it was attributed to the bacteriocin charge at low pH, it can help the bacteriocin transportation through the cell wall. However, it was found that the optimum pH for the nisin activity was 6.0 for *L. monocytogenes* [16].

Sodium chloride had the weakest effect on nisin activity compared with the rest of the factors considered in this work. The two concentrations analyzed (1 and 2 % w/v) had no effect on nisin activity against *L. fructivorans* since no differences were detected between control systems and the NaCl treatments. These results are in agreement with Castro [17] who observed no detectable differences in systems with and without 2 % w/w NaCl at a nisin concentration of 500 ppm against the same bacteria.

Conclusion

These results highlight the importance of considering thermal treatment and pH as crucial factors affecting nisin activity in food systems. Sterilization treatments considerably reduced nisin activity. *L. fructivorans* spoilage could be controlled by the use of nisin, but the concentrations of the bacteriocin have to be stablished according to the food matrix and the processing steps to which food will be exposed.

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